NEUROENDOCRINE TISSUE ENGINEERING IN ROTATING WALL VESSEL BIOREACTORS UNDER SIMULATED MICROGRAVITY CONDITIONS

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Abstract-**The low-shear, microgravity-simulating cell culture environment in Rotating Wall Vessel (RWV) Bioreactors RWV Bioreactors is well suited for generating differentiated 3-D tissue constructs (organoids). Biochemical, immunological and molecular biological techniques, including cDNA arrays, were used to analyze signal transduction, catecholamine contents, and gene expression in 3-D tissue-like constructs of PC12 pheochromocytoma cells grown for <30 d in RWVs. Vascularization of the organoids implanted s.c. into Matrigel plugs in B56 mice was evaluated by fluorescence microscopy. The unique culture environment of RWV Bioreactors facilitates the generation of macroscopic, functional neuroendocrine tissue-like assemblies, as assessed by the enhanced production of catecholamines. Organoid formation is accompanied by prolonged activation of specific signaling pathways (erk, p38, jnk) and transcription factors, leading to a unique pattern of gene expression characteristic for the neuroendocrine phenotype. Thus in RWV, "neuronal" genes (e.g. GAP 43) were downregulated while "neuroendocrine" genes (e.g. chromogranin A) were upregulated. When implanted into Matrigel plugs *in vivo*, PC12 organoids generated in RWV Bioreactors, but not 3-D aggregates formed under static conditions, became highly vascularized. This latter finding is in line with the enhanced expression of angiogenic growth factors, such as VEGF in RWVs but not in the controls. The unique cell culture environment in RWV Bioreactors activates differentiative signaling pathways and hence facilitates engineering of functional neuroendocrine tissue constructs, which might be clinically useful as implants in the treatment of debilitating neurodegenerative diseases, such as Parkinson's disease.**

Keywords- **tissue engineering; simulated microgravity; rotating wall vessel Bioreactors; neuoendocrine; signal transduction; gene expression; cDNA array**

I INTRODUCTION

Use of NASA RWV Biotechnology to Generate Differentiated 3-D Constructs: Conventional tissue culture in 2-dimensions is inadequate to model the complex cellular interactions that promote and maintain tissue-specific differentiation. Phenotypic diversity can be maintained by growing cells under conditions which provide the necessary environmental cues for organ-like assembly, for example by growing cell aggregates in suspension culture. However, the level of tissue-specific differentiation in these aggregates is limited, in part, because of the detrimental effects of the high fluid shear stress encountered in conventional stirred fermentors. Some of these problems have recently been overcome by the introduction of the Rotating Wall Vessel (RWV) Bioreactor cell culture biotechnology, which achieves spatial co-location of cells cultured in suspension, through "simulated microgravity (μG)", i.e., randomized gravitational vectors (10^{-2} g) and very low shear stress, *viz.* < 0.5 dynes/cm^2 ([1]. Cell culture in the RWV Bioreactors has significantly advanced our capabilities to create macroscopic three-dimensional tissue assemblies. Several groups have shown that the cell culture conditions in the RWV Bioreactors facilitate the assembly of tissue-like 3-D constructs and induce tissue-specific differentiation in a variety of normal and transformed cell types [2,3.

Functionally, 3-D constructs obtained from both homotypic and heterotypic cell cultures maintained in the RWV Bioreactors, express tissue-specific markers and synthesize "products", such as soluble differentiation markers, in an organ-specific fashion [4]. Tissue- specific differentiation of the 3-D constructs is enhanced in the presence of diverse cell types in heterotypic co-cultures [2]. Thus, the culture conditions in the RWV provide an excellent *in vitro* system for studying the effects of environmental cues on tissue-specific cell assembly and differentiation.

PC12 cells as model for neuroendocrine differentiation: PC12 pheochromocytoma cells, which are derived from a tumor of rat adrenal chromaffin cells, are an established model system for the bipotential differentiation of neural crest-derived cells of the sympathoadrenal lineage [5]. Depending on microenvironmental cues, cells of this lineage can differentiate in the presence of neurotrophins (such as NGF) into either sympathetic neurons or, in the presence of glucocorticoids (such as dexamethasone) or other differentiative agents into the neuroendocrine, chromaffin cell phenotype. Being bipotential as well as easily available, PC12 cells have become one of the best studied model systems for elucidating signaling pathways associated with neuronal and neuroendocrine differentiation [6].

Report Documentation Page

Report Date	Report Type	Dates Covered (from... to)
25 Oct 2001	N/A	-

Title and Subtitle Neuroendocrine Tissue Engineering in Rotating Wall Vessel Bioreactors Under Simulated Microgravity Conditions	**Contract Number**
	Grant Number
	Program Element Number
Author(s)	**Project Number**
	Task Number
	Work Unit Number
Performing Organization Name(s) and Address(es) Department Medicine Department Zoology University of Wisconsin Madison, WI	**Performing Organization Report Number**
Sponsoring/Monitoring Agency Name(s) and Address(es) US Army Research, Development & Standardization Group (UK) PSC 803 Box 15 FPO AE 09499-1500	**Sponsor/Monitor's Acronym(s)**
	Sponsor/Monitor's Report Number(s)

Distribution/Availability Statement
Approved for public release, distribution unlimited

Supplementary Notes
Papers from 23rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society, October 25-28, 2001, held in Istanbul, Turkey. See also ADM001351 for entire conference on cd-rom.

Abstract

Subject Terms

Report Classification unclassified	**Classification of this page** unclassified
Classification of Abstract unclassified	**Limitation of Abstract** UU

Number of Pages
4

The extent of either sympathetic or neuroendocrine differentiation, can be assessed by the level of expression of well defined morphological and biochemical/molecular markers. For example, neuronal differentiation is accompanied by a decrease in the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme of the catecholamine synthesizing cascade, as well as by a reduced expression of proteins involved in neurotransmission. Conversely, neuroendocrine differentiation is accompanied by enhanced expression of (neuro) peptides contained in storage granules, such as enkephalins and chromogranin), an increase in the expression of the catecholamine synthesizing enzymes, viz. TH, and, in particular, of phenylethanolamine-N-methyl transferase (PNMT), and consequently. an increase in the cellular catecholamine contents

A persistent problem of engineering replacement organs is the generation of a vascular network in the neo-tissues, a *sine qua non* for obtaining full functionality. Past attempts at generating these tissues through co-culturing in RWV Bioreactors parenchymal and vascular wall cells has resulted in enhanced tissue specific differentiation of the parenchyma. However, to date these co-cultures have not yielded a functional vasculature in these otherwise functional ' "Organoids"

PC12 cells and other sympathoadrenal cells are currently being investigated as replacement and/or transplantation tissues in neurodegenerative diseases, such as Parkinson's Disease, for the management of chronic pain, and for improved motor function in aging individuals. Our long term goal is to use the microgravity environment, either simulated in Rotating Wall Vessel (RWV) Bioreactors or during space flight, to generate functional neuroendocrine 3-D constructs either vascularized from within or capable of rapid vascularization when implanted into a host. Such organoids may be useful as clinical replacement tissue in treating neurodegenerative diseases

II METHODOLOGY

Cell Culture: PC12 pheochromocytoma cells were cultured as previously described [7]. High Aspect Ratio Vessels (HARV)-type RWV Bioreactors were inoculated with PC12 cell cultures without microcarrier beads essentially as previously described [4]. Briefly, each 35-ml HARV inoculated with a total of 4 x 10^7 cells/vessel in culture medium supplemented with charcoal-stripped sera. Initial rotation speed was set at approx. 10 rpm and then gradually increased over time as the aggregates grew in size. Control cells were maintained at identical cell density in static suspension cultures (T150 flasks positioned upright).

Analysis of Microgravity-Induced Gene Expression: Steady state mRNA expression for select genes was determined using the quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique, with specific internal PCR mimics, as previously described [4]. cDNA Arrays (Atlas blots from Clontech) were used to compare gene expression profiles of cells cultured in RWV and under control conditions.

Analysis of Activation of Transcription Factors by Electrophoretic Mobility Shift Assay (EMSA): The nuclear translocation ("activation") of transcription factors, such as SP-1 and egr-1, which are involved in adrenergic differentiation, was evaluated by using the electrophoretic mobility shift assay (EMSA), according to established protocols [8].

Analysis of MAPK Activation by Western Blotting: The level of tyrosine phosphorylation, of the extracellular-receptor-regulated MAP kinase (*erk-1/2*) as well as of a "stress activated protein kinase" (SAPK), *jnk*, was analyzed by immunoprecipitation and western blotting according to the established protocols [7].

Catecholamine Analysis: Cellular catecholamines were analyzed by HPLC with electrochemical detection, as previously described [4].

Induction of Organoid Vascularization and Analysis: Organoids grown for 30 d were implanted s.c. into Matrigel plugs in B56 mice. After 7 days, FITC-labeled was injected into the tail veins and the animals sacrificed. The extent of vascularization was evaluated by fluorescence macroscopy of whole mounts and, after staining histological sections with a fluorescent lectin, griffonia simplifolica I by fluorescence microscopy.

III RESULTS AND DISCUSSION

As previously described [2-4, 9-11], PC12 cells, when maintained for up to 30 days in RWV Bioreactors, form large macroscopic tissue-like 3-D constructs. These tissue-like assemblies exhibit a neuroendocrine phenotype, both functionally and morphologically. The induction of neuroendocrine differentiation in the microgravity-simulating environment in RWV Bioreactors is a rapid process, discernible after only a few hours of culturing the cells in the RWV environment. For example, in PC12 cells maintained for up to 48 hours in RWV Bioreactors the adrenergic enzyme PNMT, but not the rate-limiting enzyme in the catecholamine-synthesizing cascade, TH is selective induction, both at the level of gene and protein. The newly induced PNMT enzyme (protein) is functional, as assessed by the production of sizeable amounts of its product, epinephrine**.** Furthermore, RWV culture induces with time also the expression of typical extracellular matrix proteins,

such as fibronectin and type iv-collagen, which are required for cell adhesion and tissue formation [11].

The rapid induction of gene expression in RWV culture suggests that critical cellular signaling mechanisms involved in gene-regulation might be sensitive to the simulated microgravity environment. To evaluate the effects of simulated microgravity in RWV Bioreactors on intracellular signaling, PC12 cells were cultured in the absence of glucocorticoids for short periods of time (up to 24 hours, *viz.,* prior to the formation of large aggregates) and then stimulated for 10 min with NGF. Control cells were maintained under identical conditions in static suspension cultures. Under static conditions basal activities (phosphorylation) of erk-*1/2* and *jnk* are low. Upon stimulation of the control cells with NGF, the activity of *erk-1/2*, but not of *jnk* is significantly increased. By contrast, after culturing the cells for 24 hours in the HARV, the activities of both *erk-1/2* and *jnk* are elevated. Upon stimulation of the cells in the RWV with NGF, the level of *and erk-1/2* activation is not measurably altered, while that of j*nk* is significantly enhanced. These results suggest that in the RWV environment, the normal pathways of NGF signaling via the conventional MAPK*/erk* cascade may be abrogated or modified.

A causal relationship between MAPK activation and PNMT expression can be established by using some of the highly specific inhibitors of the MAPK signaling pathways. Two of these compounds, PD 98059 and SB 203580 interfere with the *erk*- and *p38*-dependent pathways by blocking the kinase activities of *MEK-1* (the kinase that phosphorylates erk 1/2) and *p38*, respectively. Inhibition of *p38* but not of *MEK-1* essentially abrogated the μG -induced up-regulation of PNMT expression.

SP-1 and CRE (the cAMP responsive element) are amongst the nuclear transcription factors traditionally associated with neuroendocrine (adrenergic) differentiation in cells of the sympathoadrenal lineage. In static suspension cultures, analysis of nuclear extracts by EMSA indicates minimal nuclear translocation of SP-1 1 h after seeding, followed by a gradual up-regulation over a 24 h period. By contrast, in RWV cultures, SP-1 is present in nuclear extracts after 1 hour and this level of SP-1 is maintained for at least 24 hours. Similarly, under static conditions, CRE and GRE are barley discernible under basal, static conditions and only activated in the presence of dexamethasone. By contrast, when the cells are cultured in glucocorticoid-free medium for 4 h in a RWV Bioreactor, both GRE and CRE activated. As expected, addition of dexamethasone further enhances nuclear levels of GRE, and also to a lesser extent of CRE.
Taken together, these results suggest that the selective upregulation of PNMT gene expression in RWV Bioreactors involves some of the "established" pathways of cellular signaling, such as activation of the MAPK cascade and the nuclear translocation of certain transcription factors.

Based on these findings, we propose a working model for the possible signaling pathways affected by simulated μG. In the RWV environment *trk*-mediated neurotrophin signaling is abrogated, concomitant with the constitutive up-regulation of some of signaling pathways (*jnk/pp38)* and transcription factors (GRE, SP-1) which might be directly associated with the induction of the neuroendocrine pathway. Whether this model is also relevant in actual microgravity will has to be tested in the proposed flight experiments [11].

As discussed before, the "simulated microgravity" environment in RWV Bioreactors entails also a low-shear component, which might, in part, contribute to the observed cellular differentiation. However, the level of shear stress in RWVs might be too low to directly induce shear stress responses via "classical" shear stress response elements. Thus, when PC12 cells were cultured in conventional stirred fermentors, at the same levels of low-shear (approx. 0.5 dynes/cm^2) as experienced in RWVs, the cells did aggregate, but did not differentiate, as assessed by the lack of an organized-tissue like structure and lack of PNMT expression. Our data favor the hypothesis that the observed up-regulation of PNMT expression and alterations in signaling pathways are at least in part due to the μG-simulating-component of the unique cell culture environment in RWV Bioreactors.

Using cDNA expression array technology (ATLAS blots) we compared differential gene expression of PC12 cells in 2-D and 3-D cultures. Preliminary results suggest that after 24hr culture in RWV Bioreactors, numerous marker genes of chromaffinergic/adrenergic differentiation are upregulated in the absence of GCs, concomitantly with the down regulation of markers of the neuronal phenotype. Moreover, as yet another indicator of neuroendocrine/ chromaffinergic differentiation, responsiveness to NGF is impaired, and often abrogated in the RWV environment, but not in 3-D suspension cultures. The expression of chromogranin A and secretogranin V, two established markers for neuroendocrine differentiation, is upregulated under static conditions by dexamethasone. In suspension cultures, the patterns of chromogranin A and secretogranin V expression are essentially unchanged, although the response to dexamethasone is diminished.

In the past there have been numerous attempts at vascularizing organoids "from within" by co-culturing in RWV Bioreactors parenchymal cells with endothelial cells. To date these experiments have not yielded the hoped-for results, presumably because of a lack of a suitable 3-DF matrix, which would facilitate vasculogenesis/ angiogenesis. We sought to revert the situation and examined the potential for organoids to be vascularized "from without:" i.e. by vessels originating form a host environment upon implantation of the organoids. For these experiments organoids grown for 30 days in RWV Bioreactors were

implanted into Matrigel plugs placed subcutaneously into syngeneic B56 mice. After 7 days, the animals were injected through their tail veins with fluorescent Dextran (as marker for the vasculature) and sacrificed. The extent of vascularization was assessed through a fluorescent stereo (macroscope). In addition histological sections were stained with a murine endothelium specific lectin (Griffonia simplifolica I) and examined by fluorescence microscopy. Only organoids grown in RWV Bioreactors showed signs of abundant vascularization throughout the Matrigel plug. The reason for this is presumably, that the organoids grown in RWV Bioreactors, but not those maintained under static conditions, produce copious amounts of an angiogenic growth factor. VEGF.

IV CONCLUSION

In summary, the unique cell culture environment in RWV Bioreactors activates specific signaling pathways fosters patterns of gene expression commensurate with a differentiated, organ-specific gene/phenotype of the cells populating the organoids. Taken together with the enhanced propensity of the organoids for soliciting exuberant angiogenesis in the host, this cell culture venue facilitates engineering functional tissue constructs, which might be useful for clinical implantation.

ACKNOWLEDGEMENTS

We gratefully continued acknowledge support by grants and collaborative research contracts (to P.I.L and B.R.U) form NASA and undergraduate research fellowships form the Wisconsin Space Grant Consortium (to E.L)

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